

NOVEL LIPASES AND USES THEREOF

Field of the invention

5 The invention relates to newly identified polynucleotide sequence comprising a gene that encodes a novel lipolytic enzyme from *Aspergillus niger*. The invention features the full length nucleotide sequence of the novel gene, the cDNA sequence comprising the full length coding sequence of the novel lipolytic enzyme as well as the amino acid sequence of the full-length lipolytic enzyme and functional equivalents
10 thereof. The invention also relates to methods of using these enzymes in industrial processes and methods of diagnosing fungal infections. Also included in the invention are cells transformed with a polynucleotide according to the invention and cells wherein a lipolytic enzyme according to the invention is genetically modified to enhance its activity and/or level of expression.

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Background of the invention

Baked products such as bread are prepared from a dough which is usually made from the basic ingredients (wheat) flour, water and optionally salt. Depending on the baked
20 products, other ingredients added may be sugars, flavours etceteras. For leavened products, primarily baker's yeast is used next to chemical leavening systems such as a combination of an acid (generating compound) and bicarbonate.

In order to improve the handling properties of the dough and/or the final properties of the baked products there is a continuous effort to develop processing aids with improving
25 properties. Processing aids are defined herein as compounds that improve the handling properties of the dough and/or the final properties of the baked products. Dough properties that may be improved comprise machineability, gas retaining capability, reduced stickiness, elasticity, extensibility, moldability etcetera. Properties of the baked products that may be improved comprise loaf volume, crust crispiness, crumb texture and softness, flavour
30 relative staleness and shelf life. These dough and/or baked product improving processing aids can be divided into two groups: chemical additives and enzymes (also referred to as baking enzymes).

Yeast, enzymes and chemical additives are generally added separately to the dough. Yeast may be added as a liquid suspension, in a compressed form or as

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active dry (ADY) or instant dry yeast (IDY). The difference between these yeast formulations is the water- and yeast dry matter content. Liquid yeast has a yeast dry matter content of less than 25% (w/v). Cream yeast is a particular form of liquid yeast and has a dry matter content between 17 and 23% (w/v). Compressed yeast has a yeast dry matter content between 25-35% (w/v) while the dry yeast formulations have a yeast dry matter content between 92-98% (w/v).

Enzymes may be added in a dry, e.g. granulated form or in liquid form. The chemical additives are in most cases added in powder form. Also, processing aid compositions which are tailored to specific baking applications, may be composed of a dedicated mixture of chemical additives and enzyme.

The preparation of a dough from the ingredients and processing aids described above is well known in the art and comprises mixing of said ingredients and processing aids and one or more moulding and fermentation steps.

The preparation of baked products from such doughs is also well known in the art and may comprise molding and shaping and further fermentation of the dough followed by baking at required temperatures and baking times.

Chemical additives with improving properties comprise oxidising agents such as ascorbic acid, bromate and azodicarbonate, reducing agents such as L-cysteine and glutathione, emulsifiers acting as dough conditioners such as diacetyl tartaric esters of mono/diglycerides (DATEM), sodium stearyl lactylate (SSL) or calcium stearyl lactylate (CSL), or acting as crumb softeners such as glycerol monostearate (GMS) etceteras, fatty materials such as triglycerides (fat) or lecithin and others.

As a result of a consumer-driven need to replace the chemical additives by more natural products, several baking enzymes have been developed with dough and/or baked product improving properties and which are used in all possible combinations depending on the specific baking application conditions. Suitable enzymes include starch degrading enzymes, arabinoxylan- and other hemicellulose degrading enzymes, cellulose degrading enzymes, oxidizing enzymes, fatty material splitting enzymes, protein degrading, modifying or crosslinking enzymes.

Starch degrading enzymes are for instance endo-acting enzymes such as alpha-amylase, maltogenic amylase, pullulanase or other debranching enzymes and exo-acting enzymes that cleave off glucose (amyloglucosidase), maltose (beta-amylase), maltotriose, maltotetraose and higher oligosaccharides.

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Arabinoxylan- and other hemicellulose degrading enzymes are for instance xylanases, pentosanases, hemicellulase, arabinofuranosidase, glucanase and others.

Cellulose degrading enzymes are for instance cellulase, cellobiohydrolase and beta-glucosidase.

5 Oxidizing enzymes are for instance glucose oxidase, hexose oxidase, pyranose oxidase, sulfhydryl oxidase, lipoxygenase, laccase, polyphenol oxidases and others.

Fatty material splitting enzymes are for instance lipolytic enzymes such as triacylglycerol lipases, phospholipases (such as A₁, A₂, B, C and D) and galactolipases.

10 Protein degrading, modifying or crosslinking enzymes are for instance endo-acting proteases (serine proteases, metalloproteases, aspartyl proteases, thiol proteases), exo-acting peptidases that cleave off one amino acid, or dipeptide, tripeptide etceteras from the N-terminal (aminopeptidases) or C-terminal (carboxypeptidases) ends of the polypeptide chain, asparagines or glutamine deamidating enzymes such as deamidase and peptidoglutaminase or crosslinking enzymes such as transglutaminase.

15 Baking enzymes may conveniently be produced in microorganisms. Microbial baking enzymes are available from a variety of sources; *Bacillus spec.* are a common source of bacterial enzymes, whereas fungal enzymes are commonly produced in *Aspergillus spec.*

20 Baking enzymes may be used in a manifold of baked goods. The term "baked goods" is herein defined as to comprise bread products such as tin bread, loaves of bread, French bread as well as rolls, cakes, pies, muffins, yeast raised and cake doughnuts and the like.

25 In the above processes, it is advantageous to use baking enzymes that are obtained by recombinant DNA techniques. Such recombinant enzymes have a number of advantages over their traditionally purified counterparts. Recombinant enzymes may be produced at a low cost price, high yield, free from contaminating agents like bacteria or viruses but also free from bacterial toxins or contaminating other enzyme activities.

Object of the invention

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It is an object of the invention to provide novel polynucleotides encoding novel lipolytic enzymes with improved properties. A further object is to provide naturally and recombinantly produced lipolytic enzymes as well as recombinant strains producing these. Also fusion polypeptides are part of the invention as well as methods of making

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and using the polynucleotides and polypeptides according to the invention.

It is also an object of the invention to provide novel lipolytic enzymes, which solve at least one of the above-mentioned problems or to provide novel lipolytic enzymes, which have one or more improved properties if used in dough and/or baked products, selected from the group of increased strength of the dough, increased elasticity of the dough, increased stability of the dough, reduced stickiness of the dough, improved extensibility of the dough, improved machineability of the dough, increased volume of the baked product, improved crumb structure of the baked product, improved softness of the baked product, improved flavour of the baked product, improved anti-staling of the baked product, improved colour of the baked product, improved crust of the baked product or which have a broad substrate specificity.

Summary of the invention

The invention provides for novel polynucleotides encoding novel lipolytic enzymes. More in particular, the invention provides for polynucleotides having a nucleotide sequence that hybridises preferably under highly stringent conditions to a sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38. Consequently, the invention provides nucleic acids that are more than 40% such as about 60%, preferably 65%, more preferably 70%, even more preferably 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% homologous to the sequences selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38.

In a more preferred embodiment the invention provides for such an isolated polynucleotide obtainable from a filamentous fungus, in particular *Aspergillus niger* is preferred.

In one embodiment, the invention provides for an isolated polynucleotide comprising a nucleic acid sequence encoding a polypeptide with an amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or functional equivalents thereof.

In a further preferred embodiment, the invention provides an isolated polynucleotide encoding at least one functional domain of a polypeptide selected from

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the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or functional equivalents thereof.

In a preferred embodiment the invention provides a lipolytic enzyme gene selected from the group consisting of SEQ ID NO: 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34 and 37. In another aspect the invention provides a polynucleotide, preferably a cDNA encoding an *Aspergillus niger* lipolytic enzyme whose amino acid sequence is selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or variants or fragments of that polypeptide. In a preferred embodiment the cDNA has a sequence selected from the group consisting of SEQ ID NO: 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35 and 38 or functional equivalents thereof.

In an even further preferred embodiment, the invention provides for a polynucleotide comprising the coding sequence of the polynucleotides according to the invention, preferred is the polynucleotide sequence selected from the group consisting of SEQ ID NO: 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35 and 38.

The invention also relates to vectors comprising a polynucleotide sequence according to the invention and primers, probes and fragments that may be used to amplify or detect the DNA according to the invention.

In a further preferred embodiment, a vector is provided wherein the polynucleotide sequence according to the invention is functionally linked with regulatory sequences suitable for expression of the encoded amino acid sequence in a suitable host cell, such as *Aspergillus niger* or *Aspergillus oryzae*. The invention also provides methods for preparing polynucleotides and vectors according to the invention.

The invention also relates to recombinantly produced host cells that contain heterologous or homologous polynucleotides according to the invention.

In another embodiment, the invention provides recombinant host cells wherein the expression of a lipolytic enzyme according to the invention is significantly increased or wherein the activity of the lipolytic enzyme is increased.

In another embodiment the invention provides for a recombinantly produced host cell that contains heterologous or homologous polynucleotide according to the invention and wherein the cell is capable of producing a functional lipolytic enzyme according to the invention, preferably a cell capable of over-expressing the lipolytic enzyme according to the invention, for example an *Aspergillus* strain comprising an increased copy number of a gene or cDNA according to the invention.

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In yet another aspect of the invention, a purified polypeptide is provided. The polypeptides according to the invention include the polypeptides encoded by the polynucleotides according to the invention. Especially preferred is a polypeptide selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or functional equivalents thereof.

Accordingly, in one aspect the present invention provides a lipolytic enzyme composition containing as an active ingredient an enzyme selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or functional equivalents thereof.

In another aspect, the invention provides a method of making baked goods wherein there is incorporated into the dough used for making the baked goods one or more enzymes selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or functional equivalents thereof.

Fusion proteins comprising a polypeptide according to the invention are also within the scope of the invention. The invention also provides methods of making the polypeptides according to the invention.

The invention also relates to the use of the lipolytic enzyme according to the invention in any industrial process as described herein.

Detailed description of the invention

A lipolytic enzyme is defined herein as an enzyme exhibiting at least one and preferably two or three or four or more of the following lipolytic activities: triacylglycerol lipase, phospholipase A₁, phospholipase A₂, phospholipase B, phospholipase C, phospholipase D, lysophospholipase and galactolipase.

Polynucleotides

The present invention provides polynucleotides encoding lipolytic enzymes having an amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or functional equivalents thereof. The sequences of the seven genes encoding the lipolytic enzymes NBE028, NBE029, NBE030, NBE031, NBE032, NBE033, NBE034, NBE036, NBE038, NBE039, NBE043, NBE045 and NBE042 respectively were determined by sequencing genomic clones

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obtained from *Aspergillus niger*. The invention provides polynucleotide sequences comprising the genes encoding the lipolytic enzymes NBE028, NBE029, NBE030, NBE031, NBE032, NBE033, NBE034, NBE036, NBE038, NBE039, NBE043, NBE045 and NBE042 as well as their complete cDNA sequences and their coding sequences (Table 1). Accordingly, the invention relates to isolated polynucleotides comprising the nucleotide sequences selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38 or functional equivalents thereof.

10 Table 1.

lipolytic enzyme	Sequence (SEQ ID NO)		
	genomic	cDNA	amino acid
NBE xxx			
NBE028	1	2	3
NBE029	4	5	6
NBE030	7	8	9
NBE031	10	11	12
NBE032	13	14	15
NBE033	16	17	18
NBE034	19	20	21
NBE036	22	23	24
NBE038	25	26	27
NBE039	28	29	30
NBE043	31	32	33
NBE045	34	35	36
NBE042	37	38	39

More in particular, the invention relates to an isolated polynucleotide hybridisable under stringent conditions, preferably under highly stringent conditions, to a polynucleotide selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38. Advantageously, such polynucleotides may be obtained from filamentous fungi, in particular from *Aspergillus niger*. More specifically, the invention relates to an isolated polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 2, 4,

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5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38.

The invention also relates to an isolated polynucleotide encoding at least one functional domain of a polypeptide having an amino acid sequences selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or
5 functional equivalents thereof.

As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which may be isolated from chromosomal DNA, which include an open reading frame encoding a protein, e.g. an *Aspergillus niger* lipolytic enzyme. A gene may include coding sequences, non-coding sequences, introns and regulatory sequences.

10 Moreover, a gene refers to an isolated nucleic acid molecule as defined herein.

A nucleic acid molecule of the present invention, such as a nucleic acid molecule having the nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38 or a functional equivalent thereof, can be isolated using standard molecular
15 biology techniques and the sequence information provided herein. For example, using all or portion of the nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38 as a hybridization probe, nucleic acid molecules according to the invention can be isolated using standard hybridization and cloning techniques (e. g., as described
20 in Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual.2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Moreover, a nucleic acid molecule encompassing all or a portion of the nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11,
25 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38 can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence information contained in the nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38.

30 A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

Furthermore, oligonucleotides corresponding to or hybridisable to nucleotide sequences according to the invention can be prepared by standard synthetic techniques, e. g., using an automated DNA synthesizer.

In one preferred embodiment, an isolated nucleic acid molecule of the invention
5 comprises the nucleotide sequence shown in SEQ ID NO: 2. The sequence of SEQ ID NO: 2 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 1. This cDNA comprises the sequence encoding the *Aspergillus niger* NBE028 polypeptide as shown in SEQ ID NO: 3.

In a second preferred embodiment, an isolated nucleic acid molecule of the
10 invention comprises the nucleotide sequence shown in SEQ ID NO: 5. The sequence of SEQ ID NO: 5 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 4. This cDNA comprises the sequence encoding the *Aspergillus niger* NBE029 polypeptide as shown in SEQ ID NO: 6.

In a third preferred embodiment, an isolated nucleic acid molecule of the
15 invention comprises the nucleotide sequence shown in SEQ ID NO: 8. The sequence of SEQ ID NO: 8 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 7. This cDNA comprises the sequence encoding the *Aspergillus niger* NBE030 polypeptide as shown in SEQ ID NO: 9.

In a fourth preferred embodiment, an isolated nucleic acid molecule of the
20 invention comprises the nucleotide sequence shown in SEQ ID NO: 11. The sequence of SEQ ID NO: 11 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 10. This cDNA comprises the sequence encoding the *Aspergillus niger* NBE031 polypeptide as shown in SEQ ID NO: 12.

In a fifth preferred embodiment, an isolated nucleic acid molecule of the
25 invention comprises the nucleotide sequence shown in SEQ ID NO: 14. The sequence of SEQ ID NO: 14 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 13. This cDNA comprises the sequence encoding the *Aspergillus niger* NBE032 polypeptide as shown in SEQ ID NO: 15.

In a sixth preferred embodiment, an isolated nucleic acid molecule of the
30 invention comprises the nucleotide sequence shown in SEQ ID NO: 17. The sequence of SEQ ID NO: 17 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 16. This cDNA comprises the sequence encoding the *Aspergillus niger* NBE033 polypeptide as shown in SEQ ID NO: 18.

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In a seventh preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 20. The sequence of SEQ ID NO: 20 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 19. This cDNA comprises the sequence encoding the

5 *Aspergillus niger* NBE034 polypeptide as shown in SEQ ID NO: 21.

In a eighth preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 23. The sequence of SEQ ID NO: 23 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 22. This cDNA comprises the sequence encoding the

10 *Aspergillus niger* NBE034 polypeptide as shown in SEQ ID NO: 24.

In a ninth preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 26. The sequence of SEQ ID NO: 26 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 25. This cDNA comprises the sequence encoding the

15 *Aspergillus niger* NBE034 polypeptide as shown in SEQ ID NO: 27.

In a tenth preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 29. The sequence of SEQ ID NO: 29 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 28. This cDNA comprises the sequence encoding the

20 *Aspergillus niger* NBE034 polypeptide as shown in SEQ ID NO: 30.

In a eleventh preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 32. The sequence of SEQ ID NO: 32 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 31. This cDNA comprises the sequence encoding the

25 *Aspergillus niger* NBE034 polypeptide as shown in SEQ ID NO: 33.

In a twelfth preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 35. The sequence of SEQ ID NO: 35 corresponds to the coding region of the *Aspergillus niger* gene provided in SEQ ID NO: 34. This cDNA comprises the sequence encoding the

30 *Aspergillus niger* NBE034 polypeptide as shown in SEQ ID NO: 36.

In a thirteenth preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO: 38. The sequence of SEQ ID NO: 38 corresponds to the coding region of the *Aspergillus niger* gene

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provided in SEQ ID NO: 37. This cDNA comprises the sequence encoding the *Aspergillus niger* NBE034 polypeptide as shown in SEQ ID NO: 39.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38 or a functional equivalent of these nucleotide sequences. A nucleic acid molecule, which is complementary to another nucleotide sequence, is one that is sufficiently complementary to the other nucleotide sequence such that it can hybridize to the other nucleotide sequence thereby forming a stable duplex.

One aspect of the invention pertains to isolated nucleic acid molecules that encode a polypeptide of the invention or a functional equivalent thereof such as a biologically active fragment or domain, as well as nucleic acid molecules sufficient for use as hybridisation probes to identify nucleic acid molecules encoding a polypeptide of the invention and fragments of such nucleic acid molecules suitable for use as PCR primers for the amplification or mutation of nucleic acid molecules. An "isolated polynucleotide" or "isolated nucleic acid" is a DNA or RNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, in one embodiment, an isolated nucleic acid includes some or all of the 5' non-coding (e.g., promotor) sequences that are immediately contiguous to the coding sequence. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide that is substantially free of cellular material, viral material, or culture medium (when produced by recombinant DNA techniques), or chemical precursors or other chemicals (when chemically synthesized). Moreover, an "isolated nucleic acid fragment" is a nucleic acid fragment that is not naturally occurring as a fragment and would not be found in the natural state.

As used herein, the terms "polynucleotide" or "nucleic acid molecule" are

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intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The nucleic acid may be synthesized using oligonucleotide
5 analogs or derivatives (e.g., inosine or phosphorothioate nucleotides). Such oligonucleotides can be used, for example, to prepare nucleic acids that have altered base-pairing abilities or increased resistance to nucleases.

Another embodiment of the invention provides an isolated nucleic acid molecule which is antisense to a nucleic acid molecule according to the invention. Also included
10 within the scope of the invention are the complement strands of the nucleic acid molecules described herein.

Sequencing errors

15 The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The specific sequences disclosed herein can be readily used to isolate the complete gene from filamentous fungi, in particular *Aspergillus niger* which in turn can easily be subjected to further sequence analyses thereby identifying sequencing errors.

20 Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any
25 nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art.
30 As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid

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sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

The person skilled in the art is capable of identifying such erroneously identified bases and knows how to correct for such errors.

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Nucleic acid fragments, probes and primers

A nucleic acid molecule according to the invention may comprise only a portion or a fragment of the nucleic acid sequence selected from the group consisting of SEQ ID
10 NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38, for example a fragment which can be used as a probe or primer or a fragment encoding a portion of protein according to the invention. The nucleotide sequence determined from the cloning of the lipolytic enzyme gene and cDNA allows for the generation of probes and primers designed for use in identifying and/or cloning other
15 lipolytic enzyme family members, as well as lipolytic enzyme homologues from other species. The probe/primer typically comprises substantially purified oligonucleotide which typically comprises a region of nucleotide sequence that hybridizes preferably under highly stringent conditions to at least about 12 or 15, preferably about 18 or 20, preferably about 22 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 or
20 more consecutive nucleotides of a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38 or of a functional equivalent thereof.

Probes based on the nucleotide sequences provided herein can be used to detect transcripts or genomic sequences encoding the same or homologous proteins for
25 instance in other organisms. In preferred embodiments, the probe further comprises a label group attached thereto, e.g., the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme cofactor. Such probes can also be used as part of a diagnostic test kit for identifying cells that express a lipolytic enzyme protein.

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Identity & homology

The terms "homology" or "percent identity" are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the

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sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (i.e. overlapping positions) x 100).

Preferably, the two sequences are the same length.

The skilled person will be aware of the fact that several different computer programmes are available to determine the homology between two sequences. For instance, a comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (J. Mol. Biol. (48): 444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

In yet another embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity two amino acid or nucleotide sequence is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989) which has been incorporated into the ALIGN program (version 2.0) (available at: <http://vega.igh.cnrs.fr/bin/align-guess.cgi>) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for

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example, identify other family members or related sequences. Such searches can be performed using the BLASTN and BLASTX programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403—10. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, word length = 12 to obtain nucleotide sequences
5 homologous to PLP03 nucleic acid molecules of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, word length = 3 to obtain amino acid sequences homologous to PLP03 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing
10 BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., BLASTX and BLASTN) can be used. See <http://www.ncbi.nlm.nih.gov>.

Hybridisation

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As used herein, the term "hybridizing" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least about 50%, at least about 60%, at least about 70%, more preferably at least about 80%, even more preferably at least about 85% to 90%, more preferably at least 95% homologous to each
20 other typically remain hybridized to each other.

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A preferred, non-limiting example of such hybridization conditions are hybridization in 6x sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 1x SSC, 0.1 % SDS at 50°C, preferably at 55°C, preferably at 60°C and even more preferably at 65°C.

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Highly stringent conditions include, for example, hybridizing at 68°C in 5x SSC/5x Denhardt's solution / 1.0% SDS and washing in 0.2x SSC/0.1% SDS at room temperature. Alternatively, washing may be performed at 42°C.

30

The skilled artisan will know which conditions to apply for stringent and highly stringent hybridisation conditions. Additional guidance regarding such conditions is readily available in the art, for example, in Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.).

Of course, a polynucleotide which hybridizes only to a poly A sequence (such

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as the 3' terminal poly(A) tract of mRNAs), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to specifically hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

Obtaining full length DNA from other organisms

In a typical approach, cDNA libraries constructed from other organisms, e.g. filamentous fungi, in particular from the species *Aspergillus* can be screened.

For example, *Aspergillus* strains can be screened for homologous polynucleotides by Northern blot analysis. Upon detection of transcripts homologous to polynucleotides according to the invention, cDNA libraries can be constructed from RNA isolated from the appropriate strain, utilizing standard techniques well known to those of skill in the art. Alternatively, a total genomic DNA library can be screened using a probe hybridisable to a polynucleotide according to the invention.

Homologous gene sequences can be isolated, for example, by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of nucleotide sequences as taught herein.

The template for the reaction can be cDNA obtained by reverse transcription of mRNA prepared from strains known or suspected to express a polynucleotide according to the invention. The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequences of a new PLP03 nucleic acid sequence, or a functional equivalent thereof.

The PCR fragment can then be used to isolate a full length cDNA clone by a variety of known methods. For example, the amplified fragment can be labeled and used to screen a bacteriophage or cosmid cDNA library. Alternatively, the labeled fragment can be used to screen a genomic library.

PCR technology can also be used to isolate full-length cDNA sequences from other organisms. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source. A reverse transcription reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis.

The resulting RNA/DNA hybrid can then be "tailed" (e.g., with guanines) using a

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standard terminal transferase reaction, the hybrid can be digested with RNase H, and second strand synthesis can then be primed (e.g., with a poly-C primer). Thus, cDNA sequences upstream of the amplified fragment can easily be isolated. For a review of useful cloning strategies, see e.g., Sambrook et al., supra; and Ausubel et al., supra.

5

Vectors

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding a protein according to the invention or a functional equivalent thereof. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms "plasmid" and "vector" can be used interchangeably herein as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vector includes one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operatively linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for

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expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signal). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive or inducible expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in a certain host cell (e.g. tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, encoded by nucleic acids as described herein (e.g. lipolytic enzymes, mutant lipolytic enzymes, fragments thereof, variants or functional equivalents thereof, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of lipolytic enzymes in prokaryotic or eukaryotic cells. For example, a protein according to the invention can be expressed in bacterial cells such as *E. coli* and *Bacillus species*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophage, yeast episome, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. The skilled person

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will know other suitable promoters. In a specific embodiment, promoters are preferred that are capable of directing a high expression level of lipolytic enzymes in filamentous fungi. Such promoters are known in the art. The expression constructs may contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding
5 site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms
10 "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, transduction, infection, lipofection, cationic lipidmediated transfection or electroporation. Suitable methods for transforming or transfecting host cells can be found
15 in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual*, 2nd, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), Davis et al., *Basic Methods in Molecular Biology* (1986) and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may
20 integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methatrexate. A nucleic acid encoding a selectable marker is preferably
25 introduced into a host cell on the same vector as that encoding a protein according to the invention or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g. cells that have incorporated the selectable marker gene will survive, while the other cells die).

Expression of proteins in prokaryotes is often carried out in *E. coli* with vectors
30 containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, e.g. to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to

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increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety after purification of the fusion protein. Such enzymes, and their
5 cognate recognition sequences, include Factor Xa, thrombin and enterokinase.

As indicated, the expression vectors will preferably contain selectable markers. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance for culturing in *E. coli* and other bacteria.

10 Representative examples of appropriate host include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS and Bowes melanoma; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

15 Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are PWLNEO, pSV2CAT, pOG44, pZT1 and pSG available from Stratagene;
20 and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Known bacterial promoters for use in the present invention include *E. coli* lacI and lacZ promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR, PL promoters and the trp promoter, the HSV thymidine kinase promoter, the early and late
25 SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter.

Inserting an enhancer sequence into the vector may increase transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes.
30 Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma

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enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signal may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification.

Polypeptides according to the invention

The invention provides an isolated polypeptide having the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39, an amino acid sequence obtainable by expressing the polynucleotide selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38 in an appropriate host. Also, a peptide or polypeptide comprising a functional equivalent of the above polypeptides is comprised within the present invention. The above polypeptides are collectively comprised in the term "polypeptides according to the invention"

The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than seven amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus. The one-letter code of amino acids used herein is commonly known in the art and can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual*, 2nd, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)

By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced

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polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention as are native or recombinant polypeptides which have been substantially purified by any suitable technique such as, for example, the single-step purification method disclosed in Smith and Johnson, Gene 67:31-40 (1988).

5 The lipolytic enzyme according to the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most
10 preferably, high performance liquid chromatography ("HPLC") is employed for purification.

 Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast,
15 higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

20 A lipolytic enzyme according to the invention may be advantageously used in baking processes. The amount of enzyme to be added to the dough is determined empirically. It may depend on the quality of the flour used, the degree of improvement which is required, the kind of bread or baked goods, the method of preparing the dough, the proportion of other ingredients etcetera.

25

Protein fragments

 The invention also features biologically active fragments of the polypeptides according to the invention.

30 Biologically active fragments of a polypeptide of the invention include polypeptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the lipolytic enzyme (e.g., the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and

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39), which include fewer amino acids than the full length protein, and exhibit at least one biological activity of the corresponding full-length protein. Typically, biologically active fragments comprise a domain or motif with at least one activity of the corresponding full length protein.

5 A biologically active fragment of a protein of the invention can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the biological activities of the native form of a polypeptide of the invention.

10 The invention also features nucleic acid fragments which encode the above biologically active fragments of the lipolytic enzyme protein.

Fusion proteins

15 The proteins of the present invention or functional equivalents thereof, e.g., biologically active portions thereof, can be operatively linked to a non-lipolytic enzyme polypeptide (e.g., heterologous amino acid sequences) to form fusion proteins. As used herein, a lipolytic enzyme "chimeric protein" or "fusion protein" comprises a lipolytic enzyme polypeptide operatively linked to a non-lipolytic enzyme polypeptide. A "lipolytic
20 enzyme polypeptide" refers to a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39, whereas a "non-lipolytic enzyme polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the lipolytic enzyme, e.g., a protein which is different from the lipolytic enzyme and
25 which is derived from the same or a different organism. Within a lipolytic enzyme fusion protein the lipolytic enzyme polypeptide can correspond to all or a portion of a lipolytic enzyme protein. In a preferred embodiment, a lipolytic enzyme fusion protein comprises at least one biologically active fragment of a lipolytic enzyme protein. In another preferred embodiment, a lipolytic enzyme fusion protein comprises at least two
30 biologically active portions of a lipolytic enzyme protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the lipolytic enzyme polypeptide and the non-lipolytic enzyme polypeptide are fused in-frame to each other. The non-lipolytic enzyme polypeptide can be fused to the N-terminus or C-terminus of the lipolytic enzyme

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polypeptide.

For example, in one embodiment, the fusion protein is a GST-lipolytic enzyme fusion protein in which the lipolytic enzyme sequence is fused to the C-terminus of the GST sequence. Such fusion proteins can facilitate the purification of recombinant lipolytic enzyme(s). In another embodiment, the fusion protein is a lipolytic enzyme protein containing a heterologous signal sequence at its N-terminus. In certain host cells (e.g., mammalian and Yeast host cells), expression and/or secretion of lipolytic enzyme can be increased through use of a heterologous signal sequence.

In another example, the gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (*Current Protocols in Molecular Biology*, Ausubel et al., eds., John Wiley & Sons, 1992). Other examples of eukaryotic heterologous signal sequences include the secretory sequences of melittin and human placental alkaline phosphatase (Stratagene; La Jolla, California). In yet another example, useful prokaryotic heterologous signal sequences include the phoA secretory signal (Sambrook et al., *supra*) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey).

A signal sequence can be used to facilitate secretion and isolation of a protein or polypeptide of the invention. Signal sequences are typically characterized by a core of hydrophobic amino acids which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway. The signal sequence directs secretion of the protein, such as from a eukaryotic host into which the expression vector is transformed, and the signal sequence is subsequently or concurrently cleaved. The protein can then be readily purified from the extracellular medium by art recognized methods. Alternatively, the signal sequence can be linked to the protein of interest using a sequence which facilitates purification, such as with a GST domain. Thus, for instance, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in *Gentz et al, Proc. Natl. Acad. Sci. USA 86:821-824 (1989)*, for instance, hexa-histidine provides for convenient

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purification of the fusion protein. The HA tag is another peptide useful for purification which corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37:767 (1984), for instance.

Preferably, a chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g. a GST polypeptide). A lipolytic enzyme-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the lipolytic enzyme protein.

Functional equivalents

The terms "functional equivalents" and "functional variants" are used interchangeably herein. Functional equivalents of lipolytic enzyme encoding DNA are isolated DNA fragments that encode a polypeptide that exhibits a particular function of the *Aspergillus niger* lipolytic enzyme as defined herein. A functional equivalent of a lipolytic enzyme polypeptide according to the invention is a polypeptide that exhibits at least one function of an *Aspergillus niger* lipolytic enzyme as defined herein. Functional equivalents therefore also encompass biologically active fragments.

Functional protein or polypeptide equivalents may contain only conservative substitutions of one or more amino acids in the amino acid sequences selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or substitutions, insertions or deletions of non-essential amino acids. Accordingly, a non-

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essential amino acid is a residue that can be altered in the amino acid sequences selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 without substantially altering the biological function. For example, amino acid residues that are conserved among the lipolytic enzyme proteins of the present invention, are predicted to be particularly unamenable to alteration. Furthermore, amino acids conserved among the lipolytic enzyme proteins according to the present invention and other lipolytic enzymes are not likely to be amenable to alteration.

The term "conservative substitution" is intended to mean that a substitution in which the amino acid residue is replaced with an amino acid residue having a similar side chain. These families are known in the art and include amino acids with basic side chains (e.g. lysine, arginine and histidine), acidic side chains (e.g. aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), non-polar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Functional nucleic acid equivalents may typically contain silent mutations or mutations that do not alter the biological function of encoded polypeptide. Accordingly, the invention provides nucleic acid molecules encoding lipolytic enzyme proteins that contain changes in amino acid residues that are not essential for a particular biological activity. Such lipolytic enzyme proteins differ in amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 yet retain at least one biological activity. In one embodiment the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises a substantially homologous amino acid sequence of at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39.

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. et al., Science 247:1306-1310 (1990) wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The

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second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selects or screens to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require non-polar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie et al, supra, and the references cited therein.

An isolated nucleic acid molecule encoding a protein homologous to the protein selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 can be created by introducing one or more nucleotide substitutions, additions or deletions into the corresponding coding nucleotide sequences (Table 1) such that one or more amino acid substitutions, deletions or insertions are introduced into the encoded protein. Such mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

The term "functional equivalents" also encompasses orthologues of the *Aspergillus niger* lipolytic enzymes provided herein. Orthologues of the *Aspergillus niger* lipolytic enzymes are proteins that can be isolated from other strains or species and possess a similar or identical biological activity. Such orthologues can readily be identified as comprising an amino acid sequence that is substantially homologous to the amino acid sequences selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39.

As defined herein, the term "substantially homologous" refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent (e.g., with similar side chain) amino acids or nucleotides to a second amino acid or nucleotide sequence such that the first and the second amino acid or nucleotide sequences have a common domain. For example, amino acid or nucleotide sequences which contain a common domain having about 60%, preferably 65%, more preferably 70%, even more preferably 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity or more are defined herein as sufficiently identical.

Also, nucleic acids encoding other lipolytic enzyme family members, which thus have a nucleotide sequence that differs from a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25,

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26, 28, 29, 31, 32, 34, 35, 37 and 38, are within the scope of the invention. Moreover, nucleic acids encoding lipolytic enzyme proteins from different species which thus have a nucleotide sequence which differs from a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26,
5 28, 29, 31, 32, 34, 35, 37 and 38 are within the scope of the invention.

Nucleic acid molecules corresponding to variants (e.g. natural allelic variants) and homologues of the polynucleotides of the invention can be isolated based on their homology to the nucleic acids disclosed herein using the cDNAs disclosed herein or a suitable fragment thereof, as a hybridisation probe according to standard hybridisation
10 techniques preferably under highly stringent hybridisation conditions.

In addition to naturally occurring allelic variants of the *Aspergillus niger* sequences provided herein, the skilled person will recognise that changes can be introduced by mutation into the nucleotide sequences selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31,
15 32, 34, 35, 37 and 38 thereby leading to changes in the amino acid sequence of the lipolytic enzyme protein without substantially altering the function of the protein.

In another aspect of the invention, improved lipolytic enzymes are provided. Improved lipolytic enzymes are proteins wherein at least one biological activity is improved. Such proteins may be obtained by randomly introducing mutations along all or
20 part of the lipolytic enzyme coding sequence, such as by saturation mutagenesis, and the resulting mutants can be expressed recombinantly and screened for biological activity. For instance, the art provides for standard assays for measuring the enzymatic activity of lipolytic enzymes and thus improved proteins may easily be selected.

In a preferred embodiment the lipolytic enzyme has an amino acid sequence
25 selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39. In another embodiment, the lipolytic enzyme is substantially homologous to the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 and retains at least one biological activity of a polypeptide selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24,
30 27, 30, 33, 36 and 39, yet differs in amino acid sequence due to natural variation or mutagenesis as described above.

In a further preferred embodiment, the lipolytic enzyme has an amino acid sequence encoded by an isolated nucleic acid fragment capable of hybridising to a

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nucleic acid selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26, 28, 29, 31, 32, 34, 35, 37 and 38, preferably under highly stringent hybridisation conditions.

Accordingly, the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 and retains at least one functional activity of the polypeptide selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39.

In particular, the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 3 or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 6, or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 9, or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 12 or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 15, or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 18 or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 21, or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 24 or the lipolytic enzyme is a protein which comprises an amino

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acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 27, or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 30 or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 33, or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 36 or the lipolytic enzyme is a protein which comprises an amino acid sequence at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 39.

Functional equivalents of a protein according to the invention can also be identified e.g. by screening combinatorial libraries of mutants, e.g. truncation mutants, of the protein of the invention for lipolytic enzyme activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display). There are a variety of methods that can be used to produce libraries of potential variants of the polypeptides of the invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

In addition, libraries of fragments of the coding sequence of a polypeptide of the invention can be used to generate a variegated population of polypeptides for screening a subsequent selection of variants. For example, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of the coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per

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molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal and internal fragments of various sizes of the protein of interest.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations of truncation, and for screening cDNA libraries for gene products having a selected property. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of a protein of the invention (Arkin and Yourvan (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815; Delgrave et al. (1993) Protein Engineering 6(3):327-331).

It will be apparent for the person skilled in the art that DNA sequence polymorphisms that may lead to changes in the amino acid sequence of the lipolytic enzyme may exist within a given population. Such genetic polymorphisms may exist in cells from different populations or within a population due to natural allelic variation. Allelic variants may also include functional equivalents.

Fragments of a polynucleotide according to the invention may also comprise polynucleotides not encoding functional polypeptides. Such polynucleotides may function as probes or primers for a PCR reaction.

Nucleic acids according to the invention irrespective of whether they encode functional or non-functional polypeptides, can be used as hybridization probes or polymerase chain reaction (PCR) primers. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having a lipolytic enzyme activity include, inter alia, (1) isolating the gene encoding the lipolytic enzyme protein, or allelic variants thereof from a cDNA library e.g. from other organisms than *Aspergillus niger*; (2) in situ hybridization (e.g. FISH) to metaphase chromosomal spreads to provide precise

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chromosomal location of the lipolytic enzyme gene as described in Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988); (3) Northern blot analysis for detecting expression of lipolytic enzyme mRNA in specific tissues and/or cells and 4) probes and primers that can be used as a diagnostic tool to
5 analyse the presence of a nucleic acid hybridisable to the lipolytic enzyme probe in a given biological (e.g. tissue) sample.

Also encompassed by the invention is a method of obtaining a functional equivalent of a lipolytic enzyme-encoding gene or cDNA. Such a method entails obtaining a labelled probe that includes an isolated nucleic acid which encodes all or a
10 portion of the sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 or a variant thereof; screening a nucleic acid fragment library with the labelled probe under conditions that allow hybridisation of the probe to nucleic acid fragments in the library, thereby forming nucleic acid duplexes, and preparing a full-length gene sequence from the nucleic acid fragments in any labelled
15 duplex to obtain a gene related to the lipolytic enzyme gene.

In one embodiment, a nucleic acid of the invention is at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, 20, 22, 23, 25, 26,
20 28, 29, 31, 32, 34, 35, 37 and 38 or the complement thereof.

In another preferred embodiment a polypeptide of the invention is at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous to an amino acid sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39.

Host cells

In another embodiment, the invention features cells, e.g., transformed host cells or recombinant host cells that contain a nucleic acid encompassed by the invention. A
30 "transformed cell" or "recombinant cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid according to the invention. Both prokaryotic and eukaryotic cells are included, e.g., bacteria, fungi, yeast, and the like, especially preferred are cells from filamentous fungi,

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in particular *Aspergillus niger*.

A host cell can be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may facilitate optimal functioning of the protein.

Various host cells have characteristic and specific mechanisms for post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems familiar to those of skill in the art of molecular biology and/or microbiology can be chosen to ensure the desired and correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such host cells are well known in the art.

Host cells also include, but are not limited to, mammalian cell lines such as CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and choroid plexus cell lines.

If desired, the polypeptides according to the invention can be produced by a stably-transfected cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, methods for constructing such cell lines are also publicly known, e.g., in Ausubel et al. (supra).

Antibodies

The invention further features antibodies, such as monoclonal or polyclonal antibodies, that specifically bind lipolytic enzyme proteins according to the invention.

As used herein, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')₂ fragments) which are capable of specifically binding to lipolytic enzyme protein. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)). Thus, these fragments are preferred.

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the lipolytic enzyme protein or an antigenic

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fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of lipolytic enzyme protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies (or lipolytic enzyme protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al.*, *Nature* 256:495 (1975); Kohler *et al.*, *Eur. J. Immunol.* 6:511 (1976); Hammerling *et al.*, In: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures involve immunizing an animal (preferably a mouse) with a lipolytic enzyme protein antigen or, with a lipolytic enzyme protein expressing cell. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferably to employ the parent myeloma cell line (SP₂O), available from the American Type Culture Collection, Rockville, Maryland. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands *et al.* (*Gastro-enterology* 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the lipolytic enzyme protein antigen. In general, the polypeptides can be coupled to a carrier protein, such as KLH, as described in Ausubel *et al.*, *supra*, mixed with an adjuvant, and injected into a host mammal.

In particular, various host animals can be immunized by injection of a polypeptide of interest. Examples of suitable host animals include rabbits, mice, guinea pigs, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), adjuvant mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridomas producing the mAbs of this

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invention can be cultivated *in vitro* or *in vivo*.

Once produced, polyclonal or monoclonal antibodies are tested for specific recognition of a protein according to the invention or functional equivalent thereof in an immunoassay, such as a Western blot or immunoprecipitation analysis using standard techniques, e.g., as described in Ausubel et al., supra. Antibodies that specifically bind to a protein according to the invention or functional equivalents thereof are useful in the invention. For example, such antibodies can be used in an immunoassay to detect a protein according to the invention in pathogenic or non-pathogenic strains of *Aspergillus* (e.g., in *Aspergillus* extracts).

Preferably, antibodies of the invention are produced using fragments of a protein according to the invention that appear likely to be antigenic, by criteria such as high frequency of charged residues. For example, such fragments may be generated by standard techniques of PCR, and then cloned into the pGEX expression vector (Ausubel et al., supra). Fusion proteins may then be expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel, et al., supra. If desired, several (e.g., two or three) fusions can be generated for each protein, and each fusion can be injected into at least two rabbits. Antisera can be raised by injections in a series, typically including at least three booster injections. Typically, the antisera are checked for their ability to immunoprecipitate the protein according to the invention or functional equivalents thereof whereas unrelated proteins may serve as a control for the specificity of the immune reaction.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778 and 4,704,692) can be adapted to produce single chain antibodies against a protein according to the invention or functional equivalents thereof. Kits for generating and screening phage display libraries are commercially available e.g. from Pharmacia.

Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223, 409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 20791; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod.*

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Hybridomas 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J.* 12:725-734.

Polyclonal and monoclonal antibodies that specifically bind a protein according to the invention of functional equivalents thereof can be used, for example, to detect expression of a gene encoding a protein according to the invention or a functional equivalent thereof e.g. in another strain of *Aspergillus*. For example, a protein according to the invention can be readily detected in conventional immunoassays of *Aspergillus* cells or extracts. Examples of suitable assays include, without limitation, Western blotting, ELISAs, radioimmune assays, and the like.

By "specifically binds" is meant that an antibody recognizes and binds a particular antigen, e.g., a protein according to the invention, but does not substantially recognize and bind other unrelated molecules in a sample.

Antibodies can be purified, for example, by affinity chromatography methods in which the polypeptide antigen is immobilized on a resin.

An antibody directed against a polypeptide of the invention (e.g., monoclonal antibody) can be used to isolate the polypeptide by standard techniques, such as affinity chromatography or immunoprecipitation. Moreover, such an antibody can be used to detect the protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the polypeptide. The antibodies can also be used diagnostically to monitor protein levels in cells or tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen or in the diagnosis of Aspergillosis..

Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive materials include ^{125}I , ^{131}I , ^{35}S or ^3H .

Preferred epitopes encompassed by the antigenic peptide are regions that are

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located on the surface of the protein, e.g., hydrophilic regions. Hydrophobicity plots of the proteins of the invention can be used to identify hydrophilic regions.

The antigenic peptide of a protein of the invention comprises at least 7 (preferably 10, 15, 20, or 30) contiguous amino acid residues of the amino acid
5 sequence selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 and encompasses an epitope of the protein such that an antibody raised against the peptide forms a specific immune complex with the protein.

Preferred epitopes encompassed by the antigenic peptide are regions of the protein according to the invention that are located on the surface of the protein, e.g.,
10 hydrophilic regions, hydrophobic regions, alpha regions, beta regions, coil regions, turn regions and flexible regions.

Immunoassays

15 Qualitative or quantitative determination of a polypeptide according to the present invention in a biological sample can occur using any art-known method. Antibody-based techniques provide special advantages for assaying specific polypeptide levels in a biological sample.

In these, the specific recognition is provided by the primary antibody (polyclonal
20 or monoclonal) but the secondary detection system can utilize fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunocomplex is obtained.

Accordingly, the invention provides a method for diagnosing whether a certain organism is infected with *Aspergillus* comprising the steps of:

- Isolating a biological sample from said organism suspected to be infected with
25 *Aspergillus*,
- reacting said biological sample with an antibody according to the invention,
- determining whether immune complexes are formed.

Tissues can also be extracted, e.g., with urea and neutral detergent, for the liberation of protein for Western-blot or dot/slot assay. This technique can also be
30 applied to body fluids.

Other antibody-based methods useful for detecting a protein according to the invention include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, monoclonal antibodies against

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a protein according to the invention can be used both as an immunoabsorbent and as an enzyme-labeled probe to detect and quantify the protein according to the invention. The amount of protein present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. In
5 another ELISA assay, two distinct specific monoclonal antibodies can be used to detect a protein according to the invention in a biological fluid. In this assay, one of the antibodies is used as the immuno-absorbent and the other as the enzyme-labeled probe.

The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting a protein according to the
10 invention with immobilized antibody and, without washing, contacting the mixture with the labeled antibody. The "two-step" assay involves washing before contacting the mixture with the labeled antibody. Other conventional methods may also be employed as suitable. It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with
15 the component and readily removed from the sample.

Suitable enzyme labels include, for example, those from the oxidase group, which catalyze the production of hydrogen peroxide by reacting with substrate. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labelled antibody/substrate reaction.

20 Besides enzymes, other suitable labels include radioisotopes, such as iodine (^{125}I , ^{127}I), carbon (^{14}C), sulphur (^{35}S), tritium (^3H), indium (^{112}In), and technetium ($^{99\text{m}}\text{Tc}$), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Specific binding of a test compound to a protein according to the invention can be detected, for example, in vitro by reversibly or irreversibly immobilizing the protein
25 according to the invention on a substrate, e.g., the surface of a well of a 96-well polystyrene microtitre plate. Methods for immobilizing polypeptides and other small molecules are well known in the art. For example, the microtitre plates can be coated with a protein according to the invention by adding the protein in a solution (typically, at a concentration of 0.05 to 1 mg/ml in a volume of 1-100 μl) to each well, and incubating
30 the plates at room temperature to 37 °C for 0.1 to 36 hours. Proteins that are not bound to the plate can be removed by shaking the excess solution from the plate, and then washing the plate (once or repeatedly) with water or a buffer. Typically, the protein is contained in water or a buffer. The plate is then washed with a buffer that lacks the

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bound protein. To block the free protein-binding sites on the plates, the plates are blocked with a protein that is unrelated to the bound protein. For example, 300 ul of bovine serum albumin (BSA) at a concentration of 2 mg/ml in Tris-HCl is suitable. Suitable substrates include those substrates that contain a defined cross-linking chemistry (e.g., plastic substrates, such as polystyrene, styrene, or polypropylene substrates from Corning Costar Corp. (Cambridge, MA), for example). If desired, a beaded particle, e.g., beaded agarose or beaded sepharose, can be used as the substrate.

Binding of the test compound to the polypeptides according to the invention can be detected by any of a variety of artknown methods. For example, a specific antibody can be used in an immunoassay. If desired, the antibody can be labeled (e.g., fluorescently or with a radioisotope) and detected directly (see, e.g., West and McMahon, J. Cell Biol. 74:264, 1977). Alternatively, a second antibody can be used for detection (e.g., a labeled antibody that binds the Fc portion of an anti-AN97 antibody). In an alternative detection method, the protein according to the invention is labeled, and the label is detected (e.g., by labeling a protein according to the invention with a radioisotope, fluorophore, chromophore, or the like). In still another method, the protein according to the invention is produced as a fusion protein with a protein that can be detected optically, e.g., green fluorescent protein (which can be detected under UV light). In an alternative method, the protein according to the invention can be covalently attached to or fused with an enzyme having a detectable enzymatic activity, such as horse radish peroxidase, alkaline phosphatase, alpha-galactosidase, or glucose oxidase. Genes encoding all of these enzymes have been cloned and are readily available for use by those of skill in the art. If desired, the fusion protein can include an antigen, and such an antigen can be detected and measured with a polyclonal or monoclonal antibody using conventional methods. Suitable antigens include enzymes (e.g., horse radish peroxidase, alkaline phosphatase, and alpha-galactosidase) and non-enzymatic polypeptides (e.g., serum proteins, such as BSA and globulins, and milk proteins, such as caseins).

Epitopes, antigens and immunogens.

In another aspect, the invention provides a peptide or polypeptide comprising

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an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen, H. M. et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1984).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G. et al., Science 219:660-666 (1984). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer, soluble peptides, especially those containing proline residues, usually are effective. Sutcliffe et al., supra, at 661. For instance, 18 of 20 peptides designed according to these guidelines, containing 8-39 residues covering 75% of the sequence of the influenza virus hemagglutinin HA1 polypeptide chain, induced antibodies that reacted with the HA1 protein or intact virus; and 12/12 peptides from the MuLV polymerase and 18/18 from the rabies glycoprotein induced antibodies that precipitated the respective proteins.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Thus, a high proportion of hybridomas obtained by fusion of spleen cells from donors immunized with an antigen epitope-bearing peptide generally secrete antibody reactive with the native protein. Sutcliffe et al., supra, at 663. The antibodies raised by antigenic epitope bearing peptides or polypeptides are useful to detect the mimicked protein, and antibodies to different

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peptides may be used for tracking the fate of various regions of a protein precursor which undergoes posttranslation processing. The peptides and anti-peptide antibodies may be used in a variety of qualitative or quantitative assays for the mimicked protein, for instance in competition assays since it has been shown that even short peptides
5 (e.g., about 9 amino acids) can bind and displace the larger peptides in immunoprecipitation assays. See, for instance, Wilson, I.A. et al., Cell 37:767-778 at 777 (1984). The anti-peptide antibodies of the invention also are useful for purification of the mimicked protein, for instance, by adsorption chromatography using methods well known in the art.

10 Antigenic epitope-bearing peptides and polypeptides of the invention designed according to the above guidelines preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of a
15 polypeptide of the invention, containing about 30 to about 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are considered epitope-bearing peptides or polypeptides of the invention and also are useful for inducing antibodies that react with the mimicked protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial
20 solubility in aqueous solvents (i.e., the sequence includes relatively hydrophilic residues and highly hydrophobic sequences are preferably avoided); and sequences containing proline residues are particularly preferred.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means for making peptides or polypeptides including
25 recombinant means using nucleic acid molecules of the invention. For instance, a short epitope-bearing amino acid sequence may be fused to a larger polypeptide which acts as a carrier during recombinant production and purification, as well as during immunization to produce anti-peptide antibodies.

Epitope-bearing peptides also may be synthesized using known methods of
30 chemical synthesis. For instance, Houghten has described a simple method for synthesis of large numbers of peptides, such as 10-20 mg of 248 different 13 residue peptides representing single amino acid variants of a segment of the HAI polypeptide which were prepared and characterized (by ELISA-type binding studies) in less than four

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weeks. Houghten, R. A., Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten et al. (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods.

A completely manual procedure allows 500-1000 or more syntheses to be conducted simultaneously. Houghten et al., supra, at 5134.

Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. See, for instance, Sutcliffe et al., supra; Wilson et al., supra; Chow, M. et al., Proc. Natl. Acad. Sci. USA 82:910-914; and Bittle, F.J. et al., J. Gen. Virol. 66:2347-2354 (1985).

Generally, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemocyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine may be coupled to carrier using a linker such as maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carrier using a more general linking agent such as glutaraldehyde.

Animals such as rabbits, rats and mice are immunized with either free or carriercoupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 ug peptide or carrier protein and Freund's adjuvant. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

Immunogenic epitope-bearing peptides of the invention, i.e., those parts of a protein that elicit an antibody response when the whole protein is the immunogen, are identified according to methods known in the art. For instance, Geysen et al., 1984, supra, discloses a procedure for rapid concurrent synthesis on solid supports of hundreds of peptides of sufficient purity to react in an enzyme-linked immunosorbent assay. Interaction of synthesized peptides with antibodies is then easily detected without

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removing them from the support. In this manner a peptide bearing an immunogenic epitope of a desired protein may be identified routinely by one of ordinary skill in the art. For instance, the immunologically important epitope in the coat protein of foot-and-mouth disease virus was located by Geysen et al. with a resolution of seven amino acids by
5 synthesis of an overlapping set of all 208 possible hexapeptides covering the entire 213 amino acid sequence of the protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope were synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. Thus, peptide analogs of the epitope-bearing peptides of
10 the invention can be made routinely by this method. U.S. Patent No. 4,708,781 to Geysen (1987) further describes this method of identifying a peptide bearing an immunogenic epitope of a desired protein.

Further still, U.S. Patent No. 5,194,392 to Geysen (1990) describes a general method of detecting or determining the sequence of monomers (amino acids or other
15 compounds) which is a topological equivalent of the epitope (i.e., a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Patent No. 4,433,092 to Geysen (1989) describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of
20 interest. Similarly, U.S. Patent No. 5,480,971 to Houghten, R. A. et al. (1996) on Peralkylated Oligopeptide Mixtures discloses linear C1-C7-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-
25 peptide analogs of the epitope-bearing peptides of the invention also can be made routinely by these methods.

Use of lipolytic enzymes in industrial processes

30 The invention also relates to the use of the lipolytic enzyme according to the invention in a selected number of industrial processes. Despite the long-term experience obtained with these processes, the lipolytic enzyme according to the invention features a number of significant advantages over the enzymes currently used. Depending on the

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specific application, these advantages can include aspects like lower production costs, higher specificity towards the substrate, less antigenic, less undesirable side activities, higher yields when produced in a suitable microorganism, more suitable pH and temperature ranges, better tastes of the final product as well as food grade and kosher aspects.

The present invention also relates to methods for preparing a dough or a baked product comprising incorporating into the dough an effective amount of a lipolytic enzyme of the present invention which improves one or more properties of the dough or the baked product obtained from the dough relative to a dough or a baked product in which the polypeptide is not incorporated.

The phrase "incorporating into the dough" is defined herein as adding the lipolytic enzyme according to the invention to the dough, any ingredient from which the dough is to be made, and/or any mixture of dough ingredients from which the dough is to be made. In other words, the lipolytic enzyme according to the invention may be added in any step of the dough preparation and may be added in one, two or more steps. The lipolytic enzyme according to the invention is added to the ingredients of a dough that is kneaded and baked to make the baked product using methods well known in the art. See, for example, U.S. Patent No. 4,567,046, EP-A-426,211, JP-A-60-78529, JP-A-62-111629, and JP-A-63-258528.

The term "effective amount" is defined herein as an amount of the lipolytic enzyme according to the invention that is sufficient for providing a measurable effect on at least one property of interest of the dough and/or baked product.

The term "improved property" is defined herein as any property of a dough and/or a product obtained from the dough, particularly a baked product, which is improved by the action of the lipolytic enzyme according to the invention relative to a dough or product in which the lipolytic enzyme according to the invention is not incorporated. The improved property may include, but is not limited to, increased strength of the dough, increased elasticity of the dough, increased stability of the dough, reduced stickiness of the dough, improved extensibility of the dough, improved flavour of the baked product, improved anti-staling of the baked product.

The improved property may be determined by comparison of a dough and/or a baked product prepared with and without addition of a polypeptide of the present invention in accordance with the methods of present invention are described below in

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the Examples. Organoleptic qualities may be evaluated using procedures well established in the baking industry, and may include, for example, the use of a panel of trained taste-testers.

5 The term "increased strength of the dough" is defined herein as the property of a dough that has generally more elastic properties and/or requires more work input to mould and shape.

The term "increased elasticity of the dough" is defined herein as the property of a dough which has a higher tendency to regain its original shape after being subjected to a certain physical strain.

10 The term "increased stability of the dough" is defined herein as the property of a dough that is less susceptible to mechanical abuse thus better maintaining its shape and volume.

15 The term "reduced stickiness of the dough" is defined herein as the property of a dough that has less tendency to adhere to surfaces, e.g., in the dough production machinery, and is either evaluated empirically by the skilled test baker or measured by the use of a texture analyser (e.g., TAXT2) as known in the art.

The term "improved extensibility of the dough" is defined herein as the property of a dough that can be subjected to increased strain or stretching without rupture.

20 The term "improved machineability of the dough" is defined herein as the property of a dough that is generally less sticky and/or more firm and/or more elastic.

The term "increased volume of the baked product" is measured as the specific volume of a given loaf of bread (volume/weight) determined typically by the traditional rapeseed displacement method.

25 The term "improved crumb structure of the baked product" is defined herein as the property of a baked product with finer and/or thinner cell walls in the crumb and/or more uniform/homogenous distribution of cells in the crumb and is usually evaluated empirically by the skilled test baker.

30 The term "improved softness of the baked product" is the opposite of "firmness" and is defined herein as the property of a baked product that is more easily compressed and is evaluated either empirically by the skilled test baker or measured by the use of a texture analyzer (e.g., TAXT2) as known in the art.

The term "improved flavor of the baked product" is evaluated by a trained test panel.

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The term "improved anti-staling of the baked product" is defined herein as the properties of a baked product that have a reduced rate of deterioration of quality parameters, e.g., softness and/or elasticity, during storage.

5 The term "dough" is defined herein as a mixture of flour and other ingredients firm enough to knead or roll. The dough may be fresh, frozen, pre-baked, or pre-baked. The preparation of frozen dough is described by Kulp and Lorenz in Frozen and Refrigerated Doughs and Batters.

10 The term "baked product" is defined herein as any product prepared from a dough, either of a soft or a crisp character. Examples of baked products, whether of a white, light or dark type, which may be advantageously produced by the present invention are bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pasta, pita bread, tortillas, tacos, cakes, pancakes, biscuits, cookies, pie crusts, steamed bread, and crisp bread, and the like.

15 Lipolytic enzyme of the present invention and/or additional enzymes to be used in the methods of the present invention may be in any form suitable for the use in question, e.g., in the form of a dry powder, agglomerated powder, or granulate, in particular a non-dusting granulate, liquid, in particular a stabilized liquid, or protected enzyme such described in WO01/11974 and WO02/26044. Granulates and agglomerated powders may be prepared by conventional methods, e.g., by spraying the
20 lipolytic enzyme according to the invention onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, e.g., a salt (such as NaCl or sodium sulphate), sugar (such as sucrose or lactose), sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy. The lipolytic enzyme according to the invention and/or additional enzymes may be contained
25 in slow-release formulations. Methods for preparing slow-release formulations are well known in the art. Adding nutritionally acceptable stabilizers such as sugar, sugar alcohol, or another polyol, and/or lactic acid or another organic acid according to established methods may for instance, stabilize liquid enzyme preparations.

30 The lipolytic enzyme according to the invention may also be incorporated in yeast comprising compositions such as disclosed in EP-A-0619947, EP-A-0659344 and WO02/49441.

For inclusion in pre-mixes of flour it is advantageous that the polypeptide according to the invention is in the form of a dry product, e.g., a non-dusting granulate,

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whereas for inclusion together with a liquid it is advantageously in a liquid form.

One or more additional enzymes may also be incorporated into the dough. The additional enzyme may be of any origin, including mammalian and plant, and preferably of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques
5 conventionally used in the art.

In a preferred embodiment, the additional enzyme may be an amylase, such as an alpha-amylase (useful for providing sugars fermentable by yeast and retarding staling) or beta-amylase, cyclodextrin glucanotransferase, peptidase, in particular, an exopeptidase (useful in flavour enhancement), transglutaminase, lipase (useful for the
10 modification of lipids present in the dough or dough constituents so as to soften the dough), phospholipase, cellulase, hemicellulase, in particular a pentosanase such as xylanase (useful for the partial hydrolysis of pentosans which increases the extensibility of the dough), protease (useful for gluten weakening in particular when using hard wheat flour), protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO
15 95/00636, glycosyltransferase, peroxidase (useful for improving the dough consistency), laccase, or oxidase, e.g., an glucose oxidase, hexose oxidase, aldose oxidase, pyranose oxidase, lipoxygenase or L-amino acid oxidase (useful in improving dough consistency).

When one or more additional enzyme activities are to be added in accordance
20 with the methods of the present invention, these activities may be added separately or together with the polypeptide according to the invention, optionally as constituent(s) of the bread-improving and/or dough-improving composition. The other enzyme activities may be any of the enzymes described above and may be dosed in accordance with established baking practices.

The present invention also relates to methods for preparing a baked product
25 comprising baking a dough obtained by a method of the present invention to produce a baked product. The baking of the dough to produce a baked product may be performed using methods well known in the art.

The present invention also relates to doughs and baked products, respectively,
30 produced by the methods of the present invention.

The present invention further relates to a pre-mix, e.g., in the form of a flour composition, for dough and/or baked products made from dough, in which the pre-mix comprises a polypeptide of the present invention. The term "pre-mix" is defined herein to

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be understood in its conventional meaning, i.e., as a mix of baking agents, generally including flour, which may be used not only in industrial bread-baking plants/facilities, but also in retail bakeries. The pre-mix may be prepared by mixing the polypeptide or a bread-improving and/or dough-improving composition of the invention comprising the polypeptide with a suitable carrier such as flour, starch, a sugar, or a salt. The pre-mix may contain other dough-improving and/or bread-improving additives, e.g., any of the additives, including enzymes, mentioned above.

The present invention further relates to baking additives in the form of a granulate or agglomerated powder, which comprise a polypeptide of the present invention. The baking additive preferably has a narrow particle size distribution with more than 95% (by weight) of the particles in the range from 25 to 500 μm .

In dough and bread making the present invention may be used in combination with the processing aids defined hereinbefore such as the chemical processing aids like oxidants (e.g. ascorbic acid), reducing agents (e.g. L-cysteine), oxidoreductases (e.g. glucose oxidase) and/or other enzymes such as polysaccharide modifying enzymes (e.g. α -amylase, hemicellulase, branching enzymes, etc.) and/or protein modifying enzymes (endoprotease, exoprotease, branching enzymes, etc.).

EXAMPLE 1

Fermentation of *Aspergillus niger*

Lipolytic enzymes encoded by the nucleotide sequence as provided herein were obtained by constructing expression plasmids containing the DNA sequences, transforming an *A. niger* strain with this plasmid and growing the *Aspergillus niger* strains in the following way.

Fresh spores (10^6 - 10^7) of *A. niger* strains were inoculated in 20 ml CSL-medium (100 ml flask, baffle) and grown for 20-24 hours at 34°C and 170 rpm. After inoculation of 5-10 ml CSL pre-culture in 100 ml CSM medium (500 ml flask, baffle) the strains were fermented at 34°C and 170 rpm for 3-5 days.

Cell-free supernatants were obtained by centrifugation in 50 ml Greiner tubes (30 minutes, 5000 rpm). The supernatants were pre-filtered over a GF/A Whatman Glass microfiber filter (150 mm \varnothing) to remove the larger particles, adjusted to pH 5 with 4 N

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KOH (if necessary) and sterile filtrated over a 0.2 μ m (bottle-top) filter with suction to remove the fungal material. The supernatants were stored at 4°C (or -20°C).

The CSL medium consisted of (in amount per litre): 100 g Corn Steep Solids (Roquette), 1 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g glucose $\cdot\text{H}_2\text{O}$ and 0.25 g Basildon (antifoam). The ingredients were dissolved in demi-water and the pH was adjusted to pH 5.8 with NaOH or H_2SO_4 ; 100 ml flasks with baffle and foam ball were filled with 20 ml fermentation broth and sterilized for 20 minutes at 120°C after which 200 μ l of a solution containing 5000 IU/ml penicillin and 5 mg/ml Streptomycin was added to each flask after cooling to room temperature.

The CSM medium consisted of (in amount per litre): 150 g maltose $\cdot\text{H}_2\text{O}$, 60 g Soytone (pepton), 1 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 15 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 g Tween 80, 0.02 g Basildon (antifoam), 20 g MES, 1 g L-arginine. The ingredients were dissolved in demi-water and the pH was adjusted to pH 6.2 with NaOH or H_2SO_4 ; 500 ml flasks with baffle and foam ball were filled with 100 ml fermentation broth and sterilized for 20 minutes at 120°C after which 1 ml of a solution containing 5000 IU/ml penicillin and 5 mg/ml Streptomycin was added to each flask after cooling to room temperature.

EXAMPLE 2

Purification of the lipolytic enzymes of the invention

Step 1 - Preparation of ultrafiltrates

The supernatants of the cultures, as obtained in Example 1, were ultrafiltrated to remove the low molecular contaminations that could interfere with the enzymatic activity determinations and the baking tests. Ultrafiltration of 30 ml supernatant was performed in a Millipore Labscale TFF system equipped with a filter with a 10 kDa cut-off.

Depending on their colour, the samples were washed 3–5 times with 40 ml volumes of cold 100 mM phosphate buffer pH 6.0 including 0.5 mM CaCl_2 . The final volume of the enzyme solution was 30 ml and is further referred to as "ultrafiltrate".

Step 2 - Determination of the lipolytic enzymes concentration by A280 and HPSEC.

The concentration of the lipolytic enzymes in the ultrafiltrate was calculated from the extinction at 280 nm (A_{280}) attributable to the lipolytic enzymes and the calculated molecular extinction coefficient of the lipolytic enzymes. Measurement of the A_{280} was

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performed in an Uvikon XL Secomam spectrophotometer (Beun de Ronde, Abcoude, The Netherlands).

The molecular extinction coefficient of an enzyme can be calculated from the number of tyrosine, tryptophan and cysteine residues per enzyme molecule (S.C. Gill and P.H. von Hippel, Anal. Biochem. 182, 319-326 (1989)). The molecular extinction coefficient of these amino acids are 1280, 5690 and 120 $M^{-1}.cm^{-1}$ respectively. The number of tyrosine, tryptophan and cysteine residues in the lipolytic enzymes of the invention can be deduced from the protein sequences selected from the group consisting of SEQ ID NO: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39. The calculated extinction coefficients of the lipolytic enzymes of the invention are summarized in Table 2.

Table 2

Lipolytic enzyme	SEQ ID NO:	# amino acids			Calculated M.W. (Da)	Calculated extinction coefficient at 280 nm	
		Trp	Tyr	Cys		$M^{-1}.cm^{-1}$	$(1\text{ mg/ml})^{-1}.cm^{-1}$
NBE028	3	13	26	6	64141	107970	1.7
NBE029	6	14	27	6	63250	114940	1.8
NBE030	9	17	26	6	59952	130730	2.2
NBE031	12	9	27	4	61173	86250	1.4
NBE032	15	3	13	6	29683	34430	1.2
NBE033	18	7	24	2	44890	70790	1.6
NBE034	21	11	19	7	53796	87750	1.6
NBE036	24	10	23	7	64945	87180	1.3
NBE038	27	13	29	4	55161	111570	2.2
NBE039	30	11	26	6	59298	96590	1.6
NBE043	33	16	35	8	62564	136800	2.2
NBE045	36	0	6	6	26688	8400	0.31
NBE042	39	14	30	7	61593	118900	1.9

15

The extinction of the ultrafiltrate at 280 nm (A_{280}) that is attributable to the lipolytic enzymes depends on the purity of the enzyme sample. This purity was

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determined using HPSEC (High Performance Size Exclusion Chromatography) with a TSK SW-XL column (300*7,8 mm; MW range 10-300 kDa). The elution buffer consisted of 25 mM sodium phosphate buffer pH 6.0 and was used at a flow of 1 ml/min. Samples of 5—100 µl were injected. The absorbance at 280 nm was measured.

- 5 The A280 in the ultrafiltrate attributable to the lipolytic enzyme of the invention was obtained from the ratio of the peak surface of the respective lipolytic enzyme peak in the chromatogram and the total surface of the peaks absorbing at 280 nm. The lipolytic enzyme concentration in the ultrafiltrate was then calculated by multiplying the A280 of the ultrafiltrate by the ratio described above and divided by the calculated extinction
10 coefficient (1 mg/ml solution – Table 2 most right column) for each lipolytic enzyme.

EXAMPLE 3

Activity measurements

- The cell-free supernatants obtained in Example 1 were subjected to the lipase,
15 phospholipase and galactolipase assays as summarized in Table 3.

Table 3. Lipolytic enzyme activities in the cell free supernatants as prepared in Example 1.

Lipolytic enzyme	Lipase	phospho lipase A	lyso phospho lipase	galacto lipase
NBE028	+	+	+	0
NBE029	+	+	+	0
NBE031	+++	+	+	+
NBE032	++	+	+	0
NBE033	+	++	+	+
NBE034	0	+	0	0
NBE036	0	+	+	0
NBE038	0	+	0	0
NBE039	+	0	0	0
NBE043	+	0	0	0

0 = not different from blanc; +/++/+++ = higher than blanc;

- 20 Lipase activity was determined spectrophotometrically by using 2,3-mercapto-1-propanol-tributyrate (TBDMP) as a substrate. Lipase hydrolyses the sulphide bond of

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TBDMP thereby liberating thio-butanoic acid which in a subsequent reaction with 4,4,-dithiodipyridine (DTDP) forms 4-thiopyridone. The latter is in a tautomeric equilibrium with 4-mercaptopyridine which absorbs at 334 nm. The reaction is carried out in 0.1 M acetate buffer pH 5.0 containing 0.2 % Triton-X100, 0.65 mM TBDMP and 0.2 mM DTDP
5 at 37°C. One lipase unit is defined as the amount of enzyme that liberates 1 micromole of 4 thio-butanoic acid per minute at the reaction conditions stated.

Phospholipase A was determined spectrophotometrically by using 1,2-dithiodioctanoyl-phosphatidylcholine as a substrate. Phospholipase A hydrolyses the
10 sulphide bond at the 1 position (PLA1) or the 2 position (PLA2) thereby liberating 4 thio-octanoic acid which, in a subsequent reaction reacts with 4,4'-dithiopyridine to form 4-thiopyridone. The latter is in tautomeric equilibrium with 4-mercaptopyridine that absorbs at 334 nm. The reaction is carried out in 0.1 M acetate buffer pH 4.0 containing 0.2 % Triton-X100, 0.65 mM substrate and 0.2 mM DTDP at 37°C. One phospholipase A unit
15 (PLA) is defined as the amount of enzyme that liberates 1 micromole of 4 thio-octanoic acid per minute at the reaction conditions stated.

Lysophospholipase activity was determined with ³¹P-NMR spectroscopy by using lysophosphatidyl-choline as a substrate. Lysophospholipase hydrolyses the ester
20 bond thereby liberating the fatty acid from the glycerol moiety. The so-formed glycerolphosphocholine is quantified using NMR.

The reaction is carried out in 50 mM acetic acid buffer pH 4.5 further containing 1 mg/ml lysophosphatidylcholine and 5 mM CaCl₂ for 30 minutes at 55°C.

One lysophospholipase unit (LPC) is defined as the amount of enzyme that forms 1
25 micromole of 4 glycerolphosphocholine per minute at the reaction conditions stated.

Galactolipase activity was determined with H-NMR spectroscopy by using digalactosyldiglyceride as a substrate, according to the method described by Hirayama and Matsuda (1972) Agric. Biol. Chem. 36, 1831. Galactolipase hydrolyses the ester
30 bond between the fatty acids and the glycerol backbone thereby liberating one or both fatty acids. The reaction is carried out in 50 mM acetic acid buffer pH 4.5 further containing 4 mM CaCl₂, 0.2% Triton X-100 and 1 mg/ml digalactosyldiglyceride (Lipid Products) for 30 minutes at 30°C. One galactolipase unit is defined as the amount of

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enzyme that forms 1 micromole of fatty acid per minute at the reaction conditions stated.

The ultrafiltrates obtained in Example 2, were subjected to the FAU enzyme activity measurement. The activity of the fungal alpha-amylase was measured using Phadebas Amylase test tablets (Pharmacia). Phadebas tablets contain a water insoluble starch substrate and a blue dye, bound by cross-linking to the substrate. The substrate is hydrolysed by fungal amylase, releasing dyed soluble maltodextrins that go into solution. A calibration curve was prepared with a solution containing a reference fungal alpha amylase activity. From the reference and unknown samples appropriate dilutions were prepared in 50 mM malic acid buffer pH 5.5. Samples of 5 ml were incubated with 30°C for 5 minutes, a Phadebas tablet was added and after 15 minutes the reaction was stopped by the addition of 1.0 ml 0.5 N sodium hydroxide. The mixtures were allowed to cool down to room temperature for 5 minutes after which 4.0 ml water was added, shaken by hand and after 15 minutes the samples were centrifuged at 4700 rpm for 10 minutes. The extinction of the top layers was measured at 620 nm. The OD 620 nm is a measure for fungal alpha amylase activity. One fungal amylase unit (FAU) is defined herein as the amount of enzyme that converts 1 gram of starch (100% dry matter) per hour into a product having a transmission at 620 nm after reaction with a iodine solution of known strength at the reaction conditions stated.

Table 4. FAU and protein in the ultrafiltrates as prepared in Example 2.

lipolytic enzyme	Protein (mg/ml) from the 280 nm analysis	fungal amylase (FAU/ml)
NBE028	2.3	4.5
NBE029	1.3	3.0
NBE030	0.4	2.6
NBE031	0.1	2.5
NBE032	1.0	0.3
NBE033	ND	0.3

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NBE034	ND	2.7
NBE036	ND	3.4
NBE038	2.0	3.7
NBE039	2.2	0.6
NBE043	0.1	0.2
NBE045	ND	4.0
NBE042	1.6	1.5

In addition to the activities mentioned in Table 4, minor activities of glucoamylase was also present, however in such low amounts that these enzymes did not interfere in the baking experiments described in example 4.

5

EXAMPLE 4

Baking experiments 1 – pup loaves

Pup loaves were baked from 150 gram dough pieces obtained by mixing 200 g flour (Kolibri™/Ibis™ in a ratio of 80/20), 1,4 g dried baker's yeast (Fermipan®), 4 g salt, 3 g sugar, 10 mg ascorbic acid, 116 g water and 2 g fat. After mixing for 6 minutes and 15 seconds in a pin mixer, the dough was divided into pieces of 150 grams and proofed for 45 minutes at 30°C, punched, proofed for another 25 minutes, moulded and panned. Proofing took place at a relative humidity of 90-100%. After a final proof of 70 minutes at 30°C, the dough was baked for 20 minutes at 225°C.

The various effects (Tables 5 and 6) of the different lipolytic enzymes in the baking experiments were compared with a control containing the same amount of fungal amylase that was added otherwise by the dosage of the ultrafiltrate (for the fungal amylase activity in the ultrafiltrates see Table 4). This was necessary since the amounts of fungal amylase added with the lipolytic enzymes in particular affected the loaf volume, not the other parameters. The volume of the breads with the control amount of fungal amylase added was taken as 100%.

Table 5.

effect		Score				
		1	2	3	4	5
Dough	dough stickiness	too sticky	sticky	control bread	much better	excellent dry
	dough extensibility	Too short	Shorter than the control	control bread	good	too long
baked bread	crumb structure	poor	non-uniform	control bread	good	excellent
	crust colour	Nearly white	too light	control bread	excellent	too dark
	crumb colour	Far too yellow	too yellow	control bread	excellent	absolutely white
	staling	Far too firm	too firm	control bread	softer	excellent

Loaf volume was determined by the Bread Volume Measurer BVM-3 (RI Cards Instruments AB, Viken, Sweden). The principle of this measurement is based on the reflection of ultrasound measured by a sensor around a rotating bread. A measurement time was taken of 45 seconds.

Dough stickiness and extensibility were evaluated by a qualified baker using the scale depicted in Table 5. The average of 2 loaves per object was measured.

After these tests the dough pieces were rounded and a first proof was performed for 45 minutes at 30°C and hereafter the dough was punched, moulded, panned, proofed for 75 minutes at 30°C. The relative humidity during the proofs was set at 85%.

Subsequently the stability of the proofed dough was judged by the presence of bladders, torn side crust and irregular curved surfaces of the crust. The dough pieces were baked for 20 minutes at 225°C. Loaf volumes were determined by the BVM-3 method: in the table the average is presented of 2 breads that are baked from the same object.

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The crumb structure was judged by a qualified baker using the scale depicted in Table 5. After storing the loaves for three days in polyethylene bags at room temperature crumb firmness was measured using a Stevens Texture Analyser. Two slices of 2 cm thickness from the centre of each loaf were analysed by the texture analyser using a probe of 1.5 inch diameter, a compression depth of 5 mm (25%) and a rate of compression of 0.5 mm/sec. In the table the average is shown of two measurements.

Crust colour was judged by a qualified baker according to the scale depicted in Table 5. As a reference the standard recipe for Dutch tin bread was used.

Crumb colour was judged by a qualified baker according to the scale depicted in Table 5. The colour of the crumb of the control breads was judged as normal (3). As a positive control the breads of 2 objects are used with the same composition as the control plus 0.5% soya flour. The proofing and baking procedure are the same as that of the control without soya flour. The latter is judged as "excellent".

The overhanging top of the bread was judged by the hanging of the top in relation to the baking tin, the lower the edges of the top the lower the judgement. The less hanging, the better the judgement,

Staling of the bread was judged by feeling the firmness of the crumb of slices of the bread. Before slicing took place, the bread was stored in a plastic bag at room temperature for 4 days. The softer the crumb of the slices is, the better the judgement.

Table 6. Baking performance of the lipolytic enzymes of the invention

Lipolytic enzyme	Parameter								
	Volume (%)	Dough stickyness	Dough extensibility	Dough stability	Crumb structure	Crust colour	Crumb colour	Overhanging top	Staling
NBE028	100	3	3	4	2	3	3	3	4
NBE029	104	3	3	4	3	4	4	4	3
NBE030	107	3	2	4	4	4	4	3	3
NBE031	102	3	2	4	5	4	4	4	4
NBE032	98	3	3	4	2	3	3	3	3

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NBE033	105	3	2	4	2	4	3	3	3
NBE034	104	3	3	4	4	4	4	4	3
NBE036	100	3	3	4	3	4	4	4	3
NBE038	109	3	3	4	5	4	4	3	3
NBE039	109	3	3	4	4	3	4	3	3
NBE043	106	3	3	4	3	4	4	3	3
NBE045	110	3	3	4	4	3	4	4	4
NBE042	110	3	4	4	4	3	4	3	3

EXAMPLE 5

Baking experiments 2 – batard

5 The baking performance of lipolytic enzymes according to the invention was tested in the French type of bread called "batard". Preparation of batards in a standard baking process was done by mixing 3000 g of wheat flour at circa 20°C, 70 g compressed yeast, 60 g salt, 68 ppm ascorbic acid, 30 ppm Bakezyme® HS₂₀₀₀ (fungal hemicellulase), 7 ppm Bakezyme® P500 (fungal α -amylase) and 1680 ml water (8–

10 10°C) in a spiral mixer (Diosna: 2 minutes in speed 1; 100 Wh input in speed 2). The dough temperature was 27°C. The machineability of the dough was analysed by hand by a baker. The dough was given a bulk proof of 15 minutes in a proofing cabinet at 32°C and 90% RH. Afterwards the dough was divided into 6 pieces of 350 g, rounded and proofed for 15 minutes at 32°C and 90% RH. At the end of this period the dough pieces

15 were moulded and shaped and given a final proof of 90 minutes at 32°C and 90% RH. The fully proofed doughs were cut in the length of the dough piece and baked in an oven at 240°C for 30 minutes with initial steam addition. After cooling down to room temperature the volumes of the loaves were determined by the BVM-method (see example 4).

20

Break, shred and shape of the breads were analysed directly after cooling down to room temperature by a qualified baker using the score in Table 7. After 16 hours (overnight) storage in a closed box at room temperature the crumb quality was assessed a qualified baker. The value for the breads (Table 8) was derived from 1 object.

Table 7

Effect		Score				
		1	2	3	4	5
Break and shred		extremely weak and soft	weak and soft	control bread	thin and crispy crust firm break of the cut	crust too thin, too hard
Crumb structure		poor	not uniform	control bread	good	excellent
shape	height	flat	medium	control bread	larger than (3)	Much larger than (3)
	cut	cut closed	cut closed	control bread	completely opened	completely opened; teared

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Table 8. Baking performance of the lipolytic enzymes of the invention

lipolytic enzyme	parameter				
	Dosage*	Loaf volume (%)	Break & Shred	Shape	Crumb structure
None	0	100	3	3	3
NBE028	0.75	3	4	4	4
NBE030	3	103	4	4	3
NBE031	2.5	95	4	4	3
NBE036	ND	88	3	3	3
NBE038	30	100	4	4	3

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NBE39	64	126	3	4	3
NBE045	ND	89	4	4	3
NBE042	12	98	3	4	4

* in ppm based on flour weight and enzyme weight determined by the A280 method